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(54) Title: GENE THERAPY VECTOR WITH OSTEOCALCIN PROMOTER AND GENES FOR BONE MORPHOGENIC PROTEINS OR GROWTH FACTORS

(57) Abstract

The present invention relates to novel compositions containing nucleotide sequences which can be used as therapeutic agents for curing a wide variety of bone and spinal disorders. More specifically, the present invention relates to novel viral vectors which are used to deliver bone growth factor genes which induce endochondral bone formation to numerous tissues. The expression of the bone growth factor genes is regulated by tissue specific and/or inducible promoters. The present invention further relates to novel methods for using the therapeutic compositions. Specifically, the methods of the invention involve administering a bone growth factor gene, either directly or systemically, to a site where bone repair and/or regeneration is necessary.

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GENE THERAPY VECTOR WITH OSTEOCALCIN PROMOTER AND GENES FOR BONE MORPHOGENIC PROTEINS OR GROWTH FACTORS

I. INTRODUCTION

The present invention relates generally to the field of gene therapy. More particularly, the present invention relates to methods and compositions related to novel, viral vectors which can be used as therapeutic agents for curing a wide variety of bone and spinal disorders. The viral vectors described in the present invention are used to deliver bone growth factor genes, which induce endochondral bone formation, to numerous tissues.

Promoters utilized with the viral vectors of the present invention are capable of selectively driving expression of a bone growth factor gene in a tissue specific manner. More particularly, the promoters are capable of selectively increasing endochondral bone formation in pre-existing bone. Thus, due to the tissue specificity of the promoters used with the viral vectors, the viral vectors of the present invention are effective therapeutic agents not only when administered via direct application, such as by injection, but also when administered systemically to the body via intravenous administration, oral administration or the like, because gene expression will be limited and localized to specific cell types.

II. BACKGROUND OF THE INVENTION

A. GENE THERAPY

Somatic cell gene therapy is a strategy in which a nucleic acid, typically in the form of DNA, is administered to alter the genetic repertoire of target cells for therapeutic purposes. Although research in experimental gene therapy is a relatively young field, major advances have been made during the last decade. (Arai, Y., *et al.,* 1997, Orthopaedic Research Society, 22:341). The potential of somatic cell gene therapy to treat human diseases has caught the imagination of numerous scientists, mainly because of two recent technologic advancements. Firstly, there are now numerous viral and non-viral gene therapy vectors that can efficiently transfer and express genes in experimental animals *in vivo.* Secondly, increasing support for the human genome project will allow for the identity and sequence of the estimated 80,000 genes comprising the human genome in the very near future.

Gene therapy was originally conceived of as a specific gene replacement therapy for correction of heritable defects to deliver functionally active therapeutic genes into targeted cells. Initial efforts toward somatic gene therapy relied on indirect means of introducing genes into tissues, called *ex vivo* gene therapy, *e.g.*, target cells are removed from the body, transfected or infected with vectors carrying recombinant genes and re-implanted into the body ("autologous cell transfer"). A variety of transfection techniques are currently available and used to transfer DNA *in vitro* into cells; including calcium phosphate-DNA precipitation, DEAE-Dextran transfection, electroporation, liposome mediated DNA transfer or transduction with recombinant viral vectors. Such *ex vivo* treatment protocols have been proposed to transfer DNA into a variety of different cell types including epithelial cells (U.S. Patent 4,868,116; Morgan and Mulligan WO87/00201; Morgan *et al.*, 1987, Science 237:1476-1479; Morgan and Mulligan, U.S. Patent No. 4,980,286), endothelial cells (WO89/05345), hepatocytes (WO89/07136; Wolff *et al.*, 1987, Proc. Natl. Acad. Sci. USA 84:3344-3348; Ledley *et al.*, 1987 Proc. Natl. Acad. Sci. 84:5335-5339; Wilson and Mulligan, WO89/07136; Wilson *et al.*, 1990, Proc. Natl. Acad. Sci. 87:8437-8441), fibroblasts (Palmer *et al.*, 1987, Proc. Natl. Acad. Sci. USA 84:1055-1059; Anson *et al.*, 1987, Mol. Biol. Med. 4:11-20; Rosenberg *et al.*, 1988, Science 242:1575-1578; Naughton & Naughton, U.S. Patent 4,963,489), lymphocytes (Anderson *et al.*, U.S. Patent No. 5,399,346; Blaese, R.M. *et al.*, 1995, Science 270:475-480) and hematopoietic stem cells (Lim, B. *et al.* 1989, Proc. Natl. Acad. Sci. USA 86:8892-8896; Anderson *et al.*, U.S. Patent No. 5,399,346).

Direct *in vivo* gene transfer recently has been attempted with formulations of DNA trapped in liposomes (Ledley *et al.*, 1987, J. Pediatrics 110:1), in proteoliposomes that contain viral envelope receptor proteins (Nicolau *et al.*, 1983, Proc. Natl. Acad. Sci. U.S.A. 80:1068) and DNA coupled to a polylysine-glycoprotein carrier complex. In addition, "gene guns" have been used for gene delivery into cells (Australian Patent No. 9068389). It even has been speculated that naked DNA, or DNA associated with liposomes, can be formulated in liquid carrier solutions for injection into interstitial spaces for transfer of DNA into cells (Felgner, WO90/11092).

Numerous clinical trials utilizing gene therapy techniques are underway for such diverse diseases as cystic fibrosis and cancer. The promise of this therapeutic approach for dramatically improving the practice of medicine has been supported widely, although

there still are many hurdles that need to be passed before this technology can be used successfully in the clinical setting.

Perhaps, one of the greatest problems associated with currently devised gene therapies, whether *ex vivo* or *in vivo*, is the inability to transfer DNA efficiently into a targeted cell population and to achieve high level expression of the gene product *in vivo*. Viral vectors are regarded as the most efficient system, and recombinant replication-defective viral vectors have been used to transduce (*i.e.*, infect) cells both *ex vivo* and *in vivo*. Such vectors have included retroviral, adenoviral, adeno-associated viral and herpes viral vectors. While highly efficient at gene transfer, the major disadvantages associated with the use of viral vectors include the inability of many viral vectors to infect non-dividing cells, problems associated with insertional mutagenesis, inflammatory reactions to the virus and potential helper virus production and/or production and transmission of harmful virus to other human patients. In addition to the low efficiency of most cell types to take up and express foreign DNA, many targeted cell populations are found in such low numbers in the body that the efficiency of presentation of DNA to the specific targeted cell types is diminished even further.

Retroviruses represent one class of viruses that have been studied extensively for use in gene therapy (Miller, A.D., 1990, Human Gene Ther. 1:5-14). Unfortunately, there are a number of disadvantages associated with retroviral use, including the random integration of retroviruses into the host genome, which often leads to insertional mutagenesis or the inadvertent activation of proto-oncogene expression due to the promoter activity associated with retroviral LTRs (long terminal repeats). Adeno-associated viruses ("AAV") also have been studied as an alternative system for delivery of stable genetic information into a cell. These viruses have the desirable feature of potentially integrating in specific regions of the host genome. However, the usefulness of both retroviral and AAV vectors is limited by their inability to accept heterologous DNA fragments greater than 3-5 Kb, their inability to produce larger quantities of viral stocks and, in the case of retroviruses, their instability and inability to infect non-dividing cells.

B. ADENOVIRUS BASED VECTORS

Adenovirus is a large, non-enveloped virus consisting of a dense protein capsid and a large linear (36 kb) double stranded DNA genome. Adenovirus infects a variety of both dividing and non-dividing cells, gaining entry by receptor-mediated uptake into endosomes, followed by internalization. After uncoating, the adenovirus genome expresses a large number of different gene products that are involved in viral replication, modification of host cell metabolism and packaging of progeny viral particles. Three adenovirus gene products are essential for replication of viral genomes: (1) the terminal binding protein which primes DNA replication, (2) the viral DNA polymerase and (3) the DNA binding protein (reviewed in Tamanoi and Stillman, 1983, Immunol. 109:75-87). In addition, processing of the terminal binding protein by the adenovirus 23kDa L3 protease is required to permit subsequent rounds of reinfection (Stillman *et al.*, 1981, Cell, 23:497-508) as well as to process adenovirus structural proteins, permitting completion of self-assembly of capsids (Bhatti and Weber, 1979, Virology, 96:478-485).

Packaging of nascent adenovirus particles takes place in the nucleus, requiring both cis-acting DNA elements and trans-acting viral factors, the latter generally construed to be a number of viral structural polypeptides. Packaging of adenoviral DNA sequences into adenovirus capsids requires the viral genomes to possess functional adenovirus encapsidation signals, which are located in the left and right termini of the linear viral genome (Hearing *et al.*, 1987, J. Virol. 61:2555-2558). Additionally, the packaging sequence must reside near the ends of the viral genome to function (Hearing *et al.*, 1987, J. Virol. 61:2555-2558; Grable and Hearing, 1992, J. Virol., 66:723-731). The E1A enhancer, the viral replication origin and the encapsidation signal compose the duplicated inverted terminal repeat (ITR) sequences located at the two ends of adenovirus genomic DNA. The replication origin is defined loosely by a series of conserved nucleotide sequences in the ITR which must be positioned close to the end of the genome to act as a replication-priming element (reviewed in Challberg and Kelly, 1989, Biochem, 58:671-717; Tamanoi and Stillman, 1983, Immunol. 109:75-87). As shown by several groups, the ITRs are sufficient to confer replication to a heterologous DNA in the presence of complementing adenovirus functions. Adenovirus "mini-chromosomes" consisting of the terminal ITRs flanking short linear DNA fragments (in some cases non-viral DNAs) were found to replicate *in vivo* at low levels in the presence of infecting wild-type adenovirus, or *in vitro* at low levels in extracts prepared from infected cells (e.g., Hay *et al.*,

1984, J. Mol. Biol. 175:493-510; Tamanoi and Stillman, 1983, Immunol. 109:75-87). Evidence for trans-packaging of mini-chromosomes was not reported in these or any later studies concerned with mechanisms of adenovirus DNA replication, and it is unlikely that packaging occurred for several reasons. First, the replicated molecules were quite small and they were not expressed at levels high enough to compete for packaging. Second, no selection for trans-packaging was employed, making it inconceivable that the heterologously replicated molecules could compete for packaging against wild-type adenovirus genomes.

The expression of foreign genes in "replication-defective" adenoviruses (deleted of region E1) has been exploited for a number of years in many labs, and a variety of published reports describe several different approaches often used in constructing these vectors (Vernon *et al.*, 1991, J. Gen. Virol., 72:1243-1251; Wilkinson and Akrigg, 1992, Nuc. Acids Res., 20:2233-2239; Eloit *et al.*, 1990, J. Gen. Virol., 71:2425-2431; Johnson, 1991; Prevec *et al.*, 1990, J. Infect. Dis., 161:27-30; Haj-Ahmad and Graham, 1986, J. Virol., 57:267-274; Lucito and Schneider, 1992, J. Virol., 66:983-991; reviewed in Graham and Prevec, 1992, Butterworth-Heinemann, 363-393). In general, replication-defective viruses are produced by replacing part, or all, of essential region E1 with a heterologous gene of interest, either by direct ligation to viral genomes *in vitro*, or by homologous recombination within cells *in vivo* (procedures reviewed in Berkner, 1992, Curr. Topics Micro. Immunol., 158:39-66). These procedures all produce adenovirus vectors that replicate in complementing cell lines such as 293 cells which provide the E1 gene products in trans. Replication competent adenovirus vectors also have been described that have the heterologous gene of interest inserted in place of non-essential region E3 (e.g., Haj-Ahmad and Graham, 1986, J. Virol. 57:267-274), or between the right ITR and region E4 (Saito *et al.*, 1985, J. Virol., 54:711-719). In both, replication defective viruses and replication competent viruses, the heterologous gene of interest is incorporated into viral particles by packaging of the recombinant adenovirus genome.

Some viral constructs, including those using retroviruses, are capable of stabile transfection of host cells, leading to long-term transgene expression. Adenoviruses, to the contrary, insert their DNA episomally, leading to transient gene expression for 2-4 weeks. For some disease processes, such as cystic fibrosis and osteoporosis, permanent transgene expression clearly would be required (Cook SD, *et al.*, 1996, Clinical Orthopedics and Related Research, 324:29-38). Thus, retroviral or adeno-associated viral vectors, which are

capable of integrating into the hosts's genome, would be desirable for the treatment of these disease processes. For other diseases, wherein transgenes encode, for example, bone growth factors, transient expression may be advantageous, since prolonged gene expression could lead to serious side-effects. In these cases, a non-integrating viral vector, such as adenovirus, would be preferred.

C. BONE MORPHOGENESIS

Defects in the process of bone repair and regeneration are linked to the development of several human diseases and disorders, *e.g.*, osteoporosis and osteogenesis imperfecta. Failure of the bone repair mechanism is, of course, associated also with significant complications in clinical orthopaedic practice, for example, during fibrous non-union following bone fracture, implant interface failures and large allograft failures. The lives of many individuals would be improved by the development of new therapies designed to stimulate and strengthen the fracture repair process.

The process of bone repair and regeneration resembles the process of wound healing in other tissues. A typical sequence of events includes; hemorrhage, clot formation, dissolution of the clot with concurrent removal of damaged tissues, ingrowth of granulation tissue, formation of cartilage, capillary ingrowth and cartilage turnover, rapid bone formation (callus tissue) and, finally, remodeling of the callus into cortical and trabecular bone. Therefore, bone repair and regeneration are complex processes that involve many cell types and regulatory molecules. The diverse cell populations involved in bone repair and regeneration include stem cells, macrophages, fibroblasts, vascular cells, osteoblasts, chondroblasts and osteoclasts.

Several currently available bone repair and regeneration therapies involve the administration of therapeutic proteins. Such therapeutic proteins may include regulatory factors such as systemic hormones, cytokines, growth factors and other proteins that regulate proliferation and differentiation of cells. Various osteoinductive agents have been purified and shown to be polypeptide growth-factor-like molecules. These stimulatory factors are referred to as bone morphogenetic or morphogenic proteins (BMPs) and also have been termed osteogenic bone inductive proteins or osteogenic proteins (OPs).

BMPs are polypeptides which induce ectopic bone formation in standard rat *in vivo* assay systems. The initial evidence suggesting that such proteins exist was first

demonstrated by Marshall Urist in the 1960s (Urist, MR, *et al.*, 1965, Science, 150:893-899). In his pioneering studies, Urist demonstrated that bone formation could be induced in extra-skeletal sites using decalcifled bone matrix and non-collagenous fractions of dentin, bone and osteosarcoma tissue. Urist's research group demonstrated that these implants stimulated pluripotent cells in the host to initiate endochondral bone formation. In 1979, Urist was the first to isolate and purify an osteoinduction factor from demineralized rat tibia matrix, which would likewise induce ectopic bone formation in rats and mice. This protein was a low molecular weight glycoprotein and confirmed the idea that individual proteins could have osteoinductive properties. The BMP field exploded in 1989, when John Wozney was successful in cloning four BMPs. Using molecular biology techniques, more than twelve such proteins now have been identified.

The BMPs are a family of proteins which belong to the TGF-β superfamily, based on amino acid similarities (Seitz, PA, *et al.*, (1992), J. Bone Min. Res., 7, 541-546). All members of the superfamily contain seven conserved cystine residues. The proteins in the TGF-β superfamily are signaling molecules which are involved with specific morphogenetic events during tissue and organ development. TGF-β is capable of transforming fibroblast cells in a monolayer culture and stimulating colony formation. It is thought that TGF-β is involved also in the control of mesenchymal cell division during development of the musculoskeletal system. BMP-2, however, seems to be involved in the differentiation of osteoblasts from pluripotent progenitor cells resident in bone marrow and extraskeletal sites.

BMPs are initially synthesized as large precursors that are approximately three times larger than the mature protein. The recombinant protein which has been studied the most heavily to date has been BMP-2, which is a 32-KD homodimeric glycoprotein. Implantation of purified recombinant BMP-2 leads to the formation of fully functional new bone, as assessed by alkaline phosphatase activity, calcium content and histology. The recombinant protein recruits nearby mesenchymal pluripotent stem cells and triggers their proliferation and differentiation into chondrocytes in less than a week. This cartilaginous tissue is then invaded by capillaries and the chondrocytes become calcified, hypertrophy and are replaced by newly formed bone in 9-12 days. During days 14-21, the mineralized bone is remodeled and is converted into a mature ossicle, filled with functional bone marrow elements. These unique properties of BMPs have stimulated great interest for their

therapeutic uses in numerous disease processes, ranging from fracture healing to osteoporosis.

As discussed, several BMP (or OP) genes now have been cloned and the common designations are BMP-1 through BMP-8. BMPs 2-8 generally are thought to be osteogenic, although BMP-1 is a more generalized morphogen (Shimell *et al.*, 1991). BMP-3 is also called osteogenin (Luyten *et al.*, 1989) and BMP-7 is also called OP-1 (Ozkaynak *et al.*, 1990). Several BMP (or OP) nucleotide sequences and polypeptides have been described in U.S. patents, *e.g.*, U.S. Pat. Nos. 4,795,804; 4,877,864; 4,968,590; 5,108,753; including, specifically, BMP-1 disclosed in U.S. Pat. No. 5,108,922; BMP-2A in U.S. Pat. Nos. 5,166,058 and 5,013,649; BMP-2B disclosed in U.S. Pat. No. 5,013,649; BMP-3 in U.S. Pat. No. 5,116,738; BMP-5 in U.S. Pat. No. 5,106,748; BMP-6 in U.S. Pat. No. 5,187,076; BMP-7 in U.S. Pat. Nos. 5,108,753 and 5,141,905; and OP-1, OP-5 and OP-7 in U.S. Pat. No. 5,011,691.

Other growth factors or hormones that have been reported to have the capacity to stimulate new bone formation include acidic fibroblast growth factor (Jingushi *et al.*, 1990), estrogen (Boden *et al.*, 1989), macrophage colony stimulating factor (Horowitz *et al.*, 1989) and calcium regulatory agents such as parathyroid hormone (PTH) (Raisz & Kream, 1983).

Several groups have investigated the possibility of using bone stimulating proteins and polypeptides, particularly recombinant BMPs, to influence bone repair *in vivo*. For example, recombinant BMP-2 has been employed to repair surgically created defects in the mandible of adult dogs (Toriumi *et al.*, 1991) and high doses of this molecule have been shown to functionally repair segmental defects in rat femurs (Yasko *et al.*, 1992). Chen and colleagues showed that a single application of 25-100 ng of recombinant TGF-β1 adjacent to cartilage induced endochondral bone formation in rabbit ear full thickness skin wounds (Chen *et al.*, 1991). It also has been reported that an application of TGF-β1 in a 3% methylcellulose gel was able to repair surgically induced large skull defects that otherwise heal by fibrous connective tissue and never form bone (Beck *et al.*, 1991).

However, a number of problems are associated with the use of therapeutic proteins, *i.e.* cytokines, in bone repair and regeneration therapies. First, the purification and/or recombinant production of therapeutic proteins often is an expensive and time-consuming process. Despite best efforts, purified protein preparations often are unstable

making storage and use cumbersome, and protein instability can lead to unexpected inflammatory reactions (to protein breakdown products) that are toxic to the host.

Second, systemic delivery of therapeutic proteins can be associated with serious unwanted side effects. Due to inefficient delivery to specific cells and tissues in the body, administration of high doses of protein are required to ensure that sufficient amounts of the protein reach the appropriate tissue target. Further, because of the short half life in the body due to proteolytic degradation, the proteins also must be administered repeatedly which may give rise to an immune reaction to the therapeutic proteins. Moreover, the circulation of high doses of therapeutic proteins often is toxic due to pleiotropic effects of the administered protein and may, thus, give rise to serious side effects..

Third, exogenous delivery of recombinant proteins is inefficient. Attempts have been made to limit the administration of high levels of protein through immobilization of therapeutic protein at the target site. However, this therapeutic approach complicates the readministration of the protein for repeated dosing.

Fourth, for a variety of proteins, such as membrane receptors, transcription factors and intracellular binding proteins, biological activity is dependant on correct expression and localization in the cell. For many proteins, correct cellular localization occurs as the protein is post-translationally modified inside the cells. Therefore, such proteins cannot be administered exogenously in such a way as to be taken up and properly localized inside the cell.

As these problems attest, it is clear that a new method capable of promoting bone repair and regeneration *in vivo* would represent a significant scientific and medical advance with immediate benefits to a large number of patients. A method readily adaptable for use with a variety of bone growth factor genes would be advantageous. Further, tissue specific expression of a bone growth factor gene, such that only specifically targeted cells and/or tissues express the bone growth factor gene, would be particularly advantageous. This is particularly true because several bone growth factors, including TGF-β, for example, have pleiotropic effects that could be extremely harmful if over-expressed in cells where bone repair and/or regeneration was not necessary.

Naturally, any new technique to stimulate bone repair and regeneration would be a valuable tool in treating bone fractures. A significant portion of fractured bones are still treated by casting, allowing natural mechanisms to effect bone repair. Although there have

been advances in fracture treatment in recent years, including improved devices, the development of new processes to stimulate, or complement, the bone repair mechanisms would represent significant progress in this, and other, related areas.

III. SUMMARY OF THE INVENTION

The present invention relates to novel compositions containing nucleotide sequences which can be used as therapeutic agents for curing a wide variety of bone and spinal disorders. The present invention further relates to novel methods for using the therapeutic compositions. More specifically, the present invention relates to novel viral vectors which are used to deliver bone growth factor genes which induce endochondral bone formation to numerous tissues. The methods of the invention further involve administering a bone growth factor gene, either directly or systemically, to a site where bone repair and/or regeneration is necessary.

The invention is based, in part, on the discovery that nucleotide sequences encoding bone growth factor genes contained within viral vectors can be administered in a cell and tissue specific manner, with the use of promoters which allow for tissue specific expression of the nucleotide sequence. An example of such a promoter is the osteocalcin promoter which is activated only within cells of osteoblastic lineage. Thus, a bone growth factor gene contained within a viral vector and controlled by an osteocalcin promoter can be expressed effectively and specifically in targeted bone cells and tissues, thereby reducing side effects of expression of the bone growth factor gene in non-osteoblastic cells.

In addition, due to the tissue specificity of the promoters used with the viral vectors, the viral vectors of the present invention are effective therapeutic agents not only when administered via direct application, such as by injection, but also when administered systemically to the body via intravenous administration, oral administration or the like, because gene expression will be limited and localized to specific, osteoblastic cell and tissue types. Further, since many of the bone growth factors of the invention, *i.e.* TGF-β, exhibit pleiotropic effects, expression of the growth factors in only specifically targeted cells is essential in order to prevent numerous, harmful side effects.

In addition to tissue specific promoters, the present invention encompasses vectors using inducible promoters. Inducible promoters have the advantage that they can be switched on and off, depending on the clinical state of the patient. Therefore, if a cell is

stably transfected with a therapeutic transgene under the control of an inducible promoter, its expression could be controlled over the life-time of an individual.

While any number of DNA sequences can be used in the methods and compositions of the present invention, preferred DNA sequences are those that encode translational products (*i.e.* proteins) or transcriptional products (*i.e.* antisense or ribozymes) that (a) promote bone repair or regeneration; or (b) are capable of disrupting a disease process (thereby allowing normal bone morphogenesis to take place).

The present invention overcomes the shortcomings of procedures currently used for bone repair or regeneration involving the administration of therapeutic proteins. First, DNA, which is both stable and non-toxic, can be administered safely in high doses *in vivo*. Second, repeated administration, while possible, is not required. The cells which take up and express the DNA provide a supply of gene product at the target site. Third, the invention could be practiced in a way that addresses the temporal requirements of dosing. For example, the DNA can be presented in vectors that integrate into the genome of the targeted cell. In this case, all daughter cells within the targeted cell will contain and express the transferred DNA, thereby acting as a continuous source for the therapeutic agent. In contrast, non-integrating systems may be utilized wherein the DNA does not integrate into the genome and the gene is not passed on to daughter cells. In such an instance, when the bone repair or regeneration process is completed and the gene product is no longer needed, the gene product will not be expressed.

The invention is demonstrated by way of examples, which show that genes can be transferred reproducibly and expressed in a variety of bone tissues *in vivo*. Specifically, it is shown that the method of the invention overcomes the problems associated with currently available gene therapy protocols. The method of the invention provides gene transfer to a suitable number of bone cells to achieve functional effects, *i.e.*, in the absence of any further targeting or cellular identification by the practitioner. *In vivo* methods of gene therapy require some form of targeting which very often does not work. In the methods of the present invention, however, targeting is not a problem since a tissue specific promoter will be utilized. Thus, expression of a nucleotide sequence, such as a bone growth factor, will occur only in osteotropic cells where the promoter is active.

In one embodiment, the method of the invention may be used as a drug delivery system through transfer of DNA into mammalian cells for the purpose of stimulating

bone repair and regeneration. The cells will be those which, when stimulated with the DNA of the invention, will express bone growth factors and thus, induce bone formation.

The DNA to be used in the practice of the invention may include any DNA encoding translational products (*i.e.* proteins) or transcriptional products (*i.e.* antisense or ribozymes) that promote bone repair or regeneration or are capable of disrupting a disease process. For example, the DNA may comprise genes encoding therapeutically useful proteins such as growth factors, cytokines, hormones, *etc.* Additionally, the DNA may encode anti-sense or ribozyme molecules that may inhibit the translation of mRNAs encoding proteins that inhibit bone repair or regeneration or which induce excessive bone morhogenesis.

The invention is illustrated by way of working examples, wherein efficient *in vivo* transfer and tissue specific expression of genes into bone tissue undergoing repair and regeneration is demonstrated.

IV. BRIEF DESCRIPTION OF THE DRAWINGS

The following drawings form part of the present specification and are included to further demonstrate certain aspects of the present invention. The invention may be understood better by reference to one or more of these drawings in combination with the detailed description of specific embodiments presented herein.

Figure 1. Osteocalcin Promoter Activity. This graph compares the Vitamin D augmentation of mouse OC (*m*OC) promoter activity in human colon (LOVO) cancer cell line with human OC (*h*OC) activity. To the right, the *h*OC promoter activity in luciferase constructs is compared to the level seen with vitamin D stimulation, which is greatly enhanced (5-100x). Thus, the human OC promoter is specific for cells of osteoblastic lineage and is induced by vitamin D.

V. DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to methods and compositions for the *in vivo* presentation and transfer of DNA into mammalian cells for the purpose of expressing therapeutic agents. The methods of the invention involve viral vectors containing genes encoding bone growth factors controlled by tissue specific and/or inducible promoters.

Bone regeneration and repair is usually a coordinated, stereotyped sequence of events that includes (a) hemorrhage; (b) clot formation; (c) dissolution of the clot with

concurrent removal of damaged tissues; (d) formation of granulation tissue (fibroplasia and angiogenesis); (e) formation of cartilage; (f) capillary ingrowth and cartilage turnover; (g) rapid bone formation (callus tissue) and (h) remodeling of the callus into cortical and trabecular bone. Therefore, bone repair and regeneration are complex processes that involve many cell types and regulatory molecules. The diverse cell populations involved in bone repair and regeneration include stem cells, macrophages, fibroblasts, vascular cells, osteoblasts, chondroblasts and osteoclasts.

The invention is based on the discovery that nucleotide sequences encoding bone growth factors contained within viral vectors can be administered in a cell and tissue specific manner, with the use of promoters which allow for tissue specific expression of the nucleotide sequences. Further, because the viral vectors of the invention utilize these promoters to control the expression of bone growth factor genes, the viral vectors of the invention are effective therapeutic agents not only when administered via direct application, but also when administered systemically to the body, because the bone growth factor genes will be expressed only in specifically targeted cells, *i.e.*, within cells of osteoblast lineage.

Taking advantage of this feature, the methods of the present invention are designed to efficiently transfer one or more DNA molecules encoding therapeutic agents to a site where bone repair and/or regeneration is necessary. The methods involve the administration of a viral vector containing DNA encoding translational products (*i.e.* therapeutic proteins) or transcriptional products (*i.e.* antisense or ribozymes) within a mammalian host to a site where bone regeneration or repair is necessary. Once the viral vector infects cells where bone repair and/or regeneration is necessary, the DNA of interest, *i.e.*, bone growth factor genes, is expressed, thereby amplifying the amount of the therapeutic agent, protein or RNA.

Alternatively, the cells may take up and express DNA encoding proteins that inhibit the activity of antagonists of the bone repair process. The DNA may encode also antisense or ribozyme RNA molecules that may be used to inhibit translation of mRNAs encoding inflammatory proteins or other factors that inhibit the bone healing process.

Stimulation of the bone repair process via direct DNA transfer from a viral vector to a mammalian cell offers a number of advantages. First, the ease of producing and purifying DNA constructs compares favorably with traditional protein production method cost. Second, direct gene transfer may be an advantageous method of drug delivery for

molecules that normally undergo complex biosynthetic processing or for receptors which must be positioned properly in the cellular membrane. These types of molecules would fail to work if delivered exogenously to cells.

The present invention relates also to pharmaceutical compositions comprising viral vectors containing DNA for use in bone repair. The compositions of the invention generally are comprised of a bio-compatible, or bone-compatible, material containing the viral vector containing DNA encoding a therapeutic protein of interest, *i.e.*, a bone growth factor. A bio-compatible composition is one that is in a form that does not produce an allergic, adverse or other untoward reaction when administered to a mammalian host.

The invention overcomes shortcomings specifically associated with current recombinant protein therapies for bone repair applications. First, direct gene transfer is a rational strategy that allows transfected cells to (a) make physiological amounts of therapeutic protein, modified in a tissue- and context-specific manner, and (b) deliver this protein to the appropriate cell surface signaling receptor under the appropriate circumstances. For reasons described above, exogenous delivery of such molecules is expected to be associated with significant dosing and delivery problems. Second, repeated administration, while possible, is not required with the methods of the invention because various promoters, including inducible promoters, can be used to control the level of expression of the therapeutic protein of interest. Further, integration of transfected DNA can be associated with long term recombinant protein expression.

A. THE NUCLEOTIDE SEQUENCES

As used herein, the terms "osteotropic and osteogenic gene" are used to refer to a gene or DNA coding region that encodes a protein, polypeptide or peptide that is capable of promoting, or assisting in the promotion of, bone formation, or one that increases the rate of primary bone growth or healing. The terms promoting, inducing and stimulating are used interchangeably throughout this text to refer to direct or indirect processes that ultimately result in the formation of new bone tissue or in an increased rate of bone repair. Thus, an osteotropic gene is a gene that, when expressed, causes the phenotype of a cell to change so that the cell either differentiates, stimulates other cells to differentiate, attracts bone-forming cells or otherwise functions in a manner that ultimately gives rise to new bone tissue.

The present methods and compositions may employ a variety of different types of DNA molecules encoding osteotropic genes. The DNA molecules may include genomic, cDNAs, single stranded DNA, double stranded DNA, triple stranded DNA, oligonucleotides and Z-DNA.

The DNA molecules may code for a variety of factors that promote bone repair including extracellular, cell surface and intracellular RNAs and proteins. Examples of extracellular proteins include growth factors, cytokines, therapeutic proteins, hormones and peptide fragments of hormones, inhibitors of cytokines, peptide growth and differentiation factors, interleukins, chemokines, interferons, colony stimulating factors and angiogenic factors. Examples of such proteins include, but are not limited to, the superfamily of TGF-β molecules, including the five TGF-β isoforms and bone morphogenetic proteins (BMP), latent TGF-β binding proteins, LTBP; keratinocyte growth factor (KGF); hepatocyte growth factor (HGF); platelet derived growth factor (PDGF); insulin-like growth factor (IGF); the basic fibroblast growth factors (FGF-1, FGF-2, *etc.*), vascular endothelial growth factor (VEGF); Factor VIII and Factor IX; erythropoietin (EPO); tissue plasminogen activator (TPA) and activins and inhibins. Hormones which may be used in the practice of the invention include, for example, growth hormone (GH) and parathyroid hormone (PTH). Examples of extracellular proteins also include the extracellular matrix proteins such as collagen, laminin and fibronectin. Examples of cell surface proteins include the family of cell adhesion molecules (*e.g.*, the integrins, selectins, Ig family members such as N-CAM and L1 and cadherins); cytokine signaling receptors such as the type I and type II TGF-β receptors and the FGF receptor and non-signaling co-receptors such as betaglycan and

syndecan. Examples of intracellular RNAs and proteins include the family of signal transducing kinases, cytoskeletal proteins such as talin and vinculin, cytokine binding proteins such as the family of latent TGF-β binding proteins and nuclear *trans* acting proteins such as transcription factors and enhancing factors.

The DNA molecules may code also for proteins that block pathological processes, thereby allowing the natural bone repair process to occur unimpeded. Examples of blocking factors include ribozymes that destroy RNA function and DNAs that, for example, code for tissue inhibitors of enzymes that destroy tissue integrity, *e.g.*, inhibitors of metalloproteinases associated with arthritis.

One may obtain the DNA segment encoding the protein of interest using a variety of molecular biological techniques, generally known to those skilled in the art. For example, cDNA or genomic libraries may be screened using primers or probes with sequences based on the known nucleotide sequences. Polymerase chain reaction (PCR) also may be used to generate the DNA fragment encoding the protein of interest. Alternatively, the DNA fragment may be obtained from a commercial source.

Genes with sequences that vary from those described in the literature also are encompassed by the invention, so long as the altered or modified gene still encodes a protein that functions to stimulate bone repair in any direct or indirect manner. These sequences include those caused by point mutations, those due to the degeneracies of the genetic code or naturally occurring allelic variants and further modifications that have been introduced by genetic engineering, *i.e.*, by the hand of man.

Techniques for introducing changes in nucleotide sequences that are designed to alter the functional properties of the encoded proteins or polypeptides are well known in the art. Such modifications include the deletion, insertion or substitution of bases which result in changes in the amino acid sequence. Changes may be made to increase the activity of an encoded protein, to increase its biological stability or half-life, to change its glycosylation pattern, to confer temperature sensitivity or to alter the expression pattern of the protein, and the like. All such modifications to the nucleotide sequences are encompassed by this invention.

The DNA encoding the translational or transcriptional products of interest may be engineered recombinantly into a variety of vector systems that provide for replication of the DNA in large scale for the preparation of the viral vectors of the invention. These vectors

can be designed to contain the necessary elements for directing the transcription and/or translation of the DNA sequence taken up by the bone cells at the repair site *in vivo*.

Vectors that may be used include, but are not limited to, those derived from recombinant bacteriophage DNA, plasmid DNA or cosmid DNA. For example, plasmid vectors such as pBR322, pUC 19/18, pUC 118, 119 and the M13 mp series of vectors may be used. Bacteriophage vectors may include λgt10, λgt11, λgt18-23, λZAP/R and the EMBL series of bacteriophage vectors. Cosmid vectors that may be utilized include, but are not limited to, pJB8, pCV 103, pCV 107, pCV 108, pTM, pMCS, pNNL, pHSG274, COS202, COS203, pWE15, pWE16 and the charomid 9 series of vectors. Vectors that allow for the *in vitro* transcription of RNA, such as SP6 vectors, also may be used to produce large quantities of RNA that may be incorporated into viral vectors.

Alternatively, recombinant virus vectors including, but not limited to, those derived from viruses such as herpes virus, retroviruses, vaccinia viruses, adenoviruses, adeno-associated viruses or bovine papilloma virus may be engineered. While integrating vectors may be used, non-integrating systems, which do not transmit the gene product to daughter cells for many generations, are preferred for non-disease related bone repair and regeneration. In this way, the gene product is expressed during the bone repair process, and as the gene is diluted out in progeny generations, the amount of expressed gene product is diminished.

Methods which are well known to those skilled in the art can be used to construct expression vectors containing the protein coding sequence operatively associated with appropriate transcriptional/translational control signals. These methods include *in vitro* recombinant DNA techniques, and synthetic techniques. *See*, for example, the techniques described in Sambrook, *et al.*, 1992, Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Laboratory, N.Y. and Ausubel *et al.*, 1989, Current Protocols in Molecular Biology, Greene Publishing Associates & Wiley Interscience, N.Y.

The genes encoding the proteins of interest may be associated operatively with a variety of different promoter/enhancer elements. The expression elements of these vectors may vary in their strength and specificities. Depending on the host/vector system utilized, any one of a number of suitable transcription and translation elements may be used. The promoter may be in the form of the promoter which is associated naturally with the gene of interest. Alternatively, the DNA may be positioned under the control of a recombinant or heterologous promoter, *i.e.*, a promoter that is not associated normally with that gene. For

example, tissue specific promoter/enhancer elements may be used to regulate the expression of the transferred DNA in specific cell types. Examples of transcriptional control regions that exhibit tissue specificity which have been described and could be used, include, but are not limited to: elastase I gene control region which is active in pancreatic acinar cells (Swift *et al.*, 1984, Cell 38:639-646; Ornitz *et al.*, 1986, Cold Spring Harbor Symp. Quant. Biol. 50:399-409; MacDonald, 1987, Hepatology 7:42S-51S); insulin gene control region which is active in pancreatic beta cells (Hanahan, 1985, Nature 315:115-122); immunoglobulin gene control region which is active in lymphoid cells (Grosschedl *et al.*, 1984, Cell 38:647-658; Adams *et al.*, 1985, Nature 318:533-538; Alexander *et al.*, 1987, Mol. Cell. Biol. 7:1436-1444); albumin gene control region which is active in liver (Pinkert *et al.*, 1987, Genes and Devel. 1:268-276); alpha-fetoprotein gene control region which is active in liver (Krumlauf *et al.*, 1985, Mol. Cell. Biol. 5:1639-1648; Hammer *et al.*, 1987, Science 235:53-58); alpha-1-antitrypsin gene control region which is active in liver (Kelsey *et al.*, 1987, Genes and Devel. 1:161-171); beta-globin gene control region which is active in myeloid cells (Magram *et al.*, 1985, Nature 315:338-340; Kollias *et al.*, 1986, Cell 46:89-94); myelin basic protein gene control region which is active in oligodendrocyte cells in the brain (Readhead *et al.*, 1987, Cell 48:703-712); myosin light chain-2 gene control region which is active in skeletal muscle (Shani, 1985, Nature 314:283-286) and gonadotropic releasing hormone gene control region which is active in the hypothalamus (Mason *et al.*, 1986, Science 234:1372-1378). Promoters isolated from the genome of viruses that grow in mammalian cells, other than the CMV promoter, (*e.g.*, RSV, vaccinia virus 7.5K, SV40, HSV, adenoviruses MLP, and MMTV LTR promoters) may be used, as well as promoters produced by recombinant DNA or synthetic techniques. Further, promoters specifically activated within bone, *i.e.*, the osteocalcin promoter, which is specifically activated within cells of osteoblastic lineage, may be used to target expression of nucleotide sequences within bone cells.

In the present invention, bone growth factor genes are expressed in host animals in a tissue specific manner. In order to obtain tissue specific expression of the bone growth factor genes, the present invention utilizes a tissue specific promoter specific for osteoblasts, the osteocalcin promoter, which can be utilized to increase bone deposition specifically in, and around, bone.

Osteocalcin (OC), a noncollagenous Gla protein produced specifically in osteoblasts, is synthesized, secreted and deposited at the time of bone mineralization (Price,

P.A., 1985, Vitam. Horm., 42:65-108). The OC promoter specifically expresses OC only in cells of osteoblastic lineage. Thus, use of viral vectors containing bone growth factor genes controlled by an OC promoter would be very effective in obtaining expression of the bone growth factor genes only in cells of osteoblastic lineage, thereby, reducing or eliminating harmful side effects caused by expression of the growth factor genes in non-osteoblastic cells. Use of universal promoters such as the CMV promoter, to the contrary, could lead to non-specific expression of the bone growth factor genes in all cells.

The use of tissue specific promoters to drive therapeutic gene expression would decrease further a toxic effect of the therapeutic gene on neighboring normal cells when virus-mediated gene delivery results in the infection of the normal cells. This would be important especially in diseases where systemic administration could be utilized to deliver a therapeutic vector throughout the body, while maintaining transgene expression to a limited and specific number of cell types. Moreover, since many bone growth factors, such as TGF-β, have pleiotropic effects, numerous, harmful side effects likely would be exhibited if the growth factor genes are expressed in all cells.

In some instances, the promoter elements may be constitutive or inducible promoters and can be used under the appropriate conditions to direct high level or regulated expression of the gene of interest. Expression of genes under the control of constitutive promoters does not require the presence of a specific substrate to induce gene expression and will occur under all conditions of cell growth. In contrast, expression of genes controlled by inducible promoters is responsive to the presence or absence of an inducing agent. For example, if a cell is stably transfected with a therapeutic, inducible transgene, its expression could be controlled over the life-time of the individual. In fact, the OC promoter, itself, is induced by vitamin D. *See,* Fig. 1. In the case of a disease such as osteoporosis, for example, bone growth factor production by bone cells could be increased whenever bone resorption becomes significant. Levels of bone resorption can be assessed easily using quantitative techniques well known to those of skill in the art.

Specific initiation signals also are required for sufficient translation of inserted protein coding sequences. These signals include the ATG initiation codon and adjacent sequences. In cases where the entire coding sequence, including the initiation codon and adjacent sequences, are inserted into the appropriate expression vectors, no additional translational control signals may be needed. However, in cases where only a portion of the

coding sequence is inserted, exogenous translational control signals, including the ATG initiation codon, must be provided. Furthermore, the initiation codon must be in phase with the reading frame of the protein coding sequences to ensure translation of the entire insert. These exogenous translational control signals and initiation codons can be of a variety of origins, both natural and synthetic. The efficiency and control of expression may be enhanced by the inclusion of transcription attenuation sequences, enhancer elements, *etc.*

In addition to DNA sequences encoding therapeutic proteins of interest, the scope of the present invention includes the use of ribozymes or antisense DNA molecules that may be transferred into mammalian cells. Such ribozymes and antisense molecules may be used to inhibit the translation of RNA encoding proteins of genes that inhibit a disease process or the bone repair process thereby allowing tissue repair to take place. Such molecules also may be used to inhibit a disease wherein over-expression of a bone growth factor gene occurs.

The expression of antisense RNA molecules will act directly to block the translation of mRNA by binding to targeted mRNA and preventing protein translation. The expression of ribozymes, which are enzymatic RNA molecules capable of catalyzing the specific cleavage of RNA, also may be used to block protein translation. The mechanism of ribozyme action involves sequence specific hybridization of the ribozyme molecule to complementary target RNA, followed by an endonucleolytic cleavage. Within the scope of the invention are engineered hammerhead motif ribozyme molecules that specifically and efficiently catalyze endonucleolytic cleavage of RNA sequences. RNA molecules may be generated by transcription of DNA sequences encoding the RNA molecule.

It also is within the scope of the invention that multiple genes, combined on a single genetic construct under control of one or more promoters, or prepared as separate constructs of the same or different types, may be used. Thus, an almost endless combination of different genes and genetic constructs may be employed. Certain gene combinations may be designed to, or their use may otherwise result in, achieving synergistic effects on bone stimulation and regeneration, any and all such combinations are intended to fall within the scope of the present invention. Indeed, many synergistic effects have been described in the scientific literature, so that one of ordinary skill in the art readily would be able to identify likely synergistic gene combinations, or even gene-protein combinations.

B. BONE REGENERATION

Bone has a substantial capacity to regenerate following fracture. The complex, but ordered, fracture repair sequence includes hemostasis, clot dissolution, granulation tissue ingrowth, formation of a callus and remodeling of the callus to an optimized structure (A.W. Ham., 1930, J. Bone Joint Surg. 12, 827-844). Cells participating in this process include platelets, inflammatory cells, fibroblasts, endothelial cells, pericytes, osteoclasts and osteogenic progenitors. Recently, several peptide growth and differentiation factors have been identified that appear to control cellular events associated with bone formation and repair (Erlebacher, A., *et al.*, 1995, Cell 80, 371-378). Bone morphogenetic proteins (BMPs), for example, are soluble extracellular factors that control osteogenic cell fate. BMP genes are normally expressed by cultured fetal osteoblasts (Harris, S.E., *et al.*, 1994, J. Bone Min. Res. 9, 389-394) and by osteoblasts during mouse embryo skeletogenesis (Lyons, K.M., *et al.*, 1989, Genes Dev. 3, 1657-1668; Lyons, K.M., *et al.*, 1990, Development 190, 833-844; Jones, M.C., *et al.*, 1991, Development 111, 531-542), recombinant BMP proteins initiate cartilage and bone progenitor cell differentiation (Yamaguchi, A., *et al.*, 1991, J. Cell Biol. 113, 681-687; Ahrens, M., *et al.*, 1993, J. Bone Min. Res. 12, 871-880; Gitelman, S.E., *et al.*, 1994, J. Cell Biol. 126, 1595-1609; Rosen, V., *et al.*, 1994, J. Cell Biol. 127, 1755-1766), delivery of recombinant BMPs induce a bone formation sequence similar to endochondral bone formation (Wozney, J.M., 1992, Mol. Reprod. Dev. 32, 160-167; Reddi, A.H., 1994, Curr. Opin. Genet. Dev. 4, 737-744) and BMP-4 gene expression is unregulated early in the process of fracture repair (Nakase, T., *et al.*, 1994, J. Bone Min. Res. 9, 651-659). Osteogenic protein-1, a member of a family of molecules related to the BMPs (Ozkaynak, E., *et al.*, 1990, EMBO J. 9, 2085-2093), is capable of similar effects *in vitro* and *in vivo* (Sampath, T.K., *et al.*, 1992, J. Biol. Chem. 267, 20352-20362; Cook, S.D., *et al.*, (1994) J. Bone Joint Surg. 76-A, 827-838). TGF-β also has been shown to stimulate cartilage and bone formation *in vivo* (Centrella, M., *et al.*, 1994, Endocrine Rev. 15, 27-38; Sumner, D.R., *et al.*, 1995, J. Bone Joint Surg. 77A, 1135-1147). Finally, parathyroid hormone (PTH) is an 84 amino acid hormone that raises the plasma and extracellular fluid Ca^{+2} concentration. In skeletal tissues, intermittent administration of a PTH fragment-possessing the structural requirements for biological activity (amino acids 1-34) produces a true anabolic effect: numerous *in vivo* and *in vitro* studies provide strong evidence that PTH1-34 administration in animals (including rats) results in uncoupled, high-

quality bone formation due to a combined inhibitory effect on osteoclasts and stimulatory effect on osteogenic cells (Dempster, D.W., *et al.*, 1993, Endocrine Rev. 14, 690-709). The PTH1-34 peptide is known to interact synergistically with BMP-4, which up-regulates the expression of functional cell surface PTH receptors in differentiating osteoblasts *in vitro* (Ahrens, M., *et al.*, 1993, J. Bone Min. Res. 12, 871-880).

As recombinant proteins, peptide growth and differentiation factors, such as BMP and TGF-β1, represent promising therapeutic alternatives for fracture repair (Wozney, J.M., 1992, Mol. Reprod. Dev. 32, 160-167; Reddi, A.H., 1994, Curr. Opin. Genet. Dev. 4, 737-744; Centrella, M., *et al.*, 1994, Endocrine Rev. 15, 27-38; Sumner, D.R., *et al.*, 1995 J. Bone Joint Surg. 77-A, 1135-1147). However, relatively large doses (microgram amounts) are required to stimulate significant new bone formation in animals, raising the concern that future human therapies may be expensive and may possess an increased risk of toxicity.

Defects in the process of bone repair and regeneration are associated with significant complications in clinical orthopaedic practice, for example, fibrous non-union following bone fracture, implant interface failures and large allograft failures. Many complex fractures currently are treated using autografts, but this technique is not effective and is associated with complications.

Naturally, any new technique designed to stimulate bone repair would be a valuable tool in treating bone fractures. A significant portion of fractured bones still are treated by casting, allowing natural mechanisms to effect wound repair. Although there have been advances in fracture treatment in recent years, including improved devices, the development of new processes to stimulate, or complement, the wound repair mechanisms would represent significant progress in this area.

The present invention may be used to transfer a bone growth factor gene to promote fracture repair. Other important aspects of this technology include the use of gene transfer to treat patents with "weak bones", such as in diseases like osteoporosis, to improve poor healing which may arise for unknown reasons, *e.g.*, fibrous non-union and to promote implant integration and the function of artificial joints.

For treatment of diseases like osteoporosis, where permanent expression of a bone-stimulating transgene is necessary, the use of integrating vectors, wherein the transgene incorporates into the host's genome, are preferred. Retroviral and adeno-associated viral vectors are ideal vectors for obtaining this type of transgene integration. However, for the

treatment of more transient conditions, such as for the healing of non-disease related fractures, the use of a non-integrating vector, wherein the bone-stimulating transgene does not get incorporated into the host's genome, is preferred. Adenovirus is a vector which is ideal for obtaining this type of non-integrated transgene expression.

Bone tissue is known to have the capacity for repair and regeneration and there is a certain understanding of the cellular and molecular basis of these processes. The initiation of new bone formation involves the commitment, clonal expansion and differentiation of repair cells. Once initiated, bone formation is promoted by a variety of polypeptide growth factors. Newly formed bone then is maintained by a series of local and systemic growth and differentiation factors.

Several bone morphogenetic protein genes now have been cloned (Wozney *et al.*, 1988; Rosen *et al.* 1989, Connect. Tissue Res., 20:313:319; summarized in Alper, 1994) and this work has established BMPs as members of the transforming growth factor-β (TGF-β) superfamily based on DNA sequence homologies. The cloning of distinct BMP genes has led to the designation of individual BMP genes and proteins as BMP-1 through at least BMP-8. BMPs 2-8 generally are thought to be osteogenic while BMP-1 may be a more generalized morphogen; Shimell *et al.*, 1991, Cell, 67:469-481). BMP-3 is also called osteogen (Luyten *et al.*, 1989, J. Biol. Chem., 264:13377-13380) and BMP-7 is also called OP-1 (Ozkaynak *et al.*, 1990, EMBO J., 9:2085-2093). TGFs and BMPs each act on cells via complex, tissue-specific interactions with families of cell surface receptors (Roberts & Sporn, 1989, M.B. Sporn and A.B. Roberts, Eds., Springer-Verlag, Heidelberg, 95 (Part 1); Aralkar *et al.*, 1991).

Transforming growth factors (TGFs) also have been shown to have a central role in regulating bone healing by affecting cell proliferation, gene expression and matrix protein synthesis (Roberts & Sporn, 1989, M.B. Sporn and A.B. Roberts, Eds., Springer-Verlag, Heidelberg, 95 (Part 1)). For example, TGF-β1 and TGF-β2 can initiate both chondrogenesis and osteogenesis (Joyce *et al.*, 1990, J. Cell Biol., 110:195-2007; Izumi *et al.*, 1992, J. Bone Min. Res., 7:115-11; Jingushi *et al.*, 1992, J. Orthop. Res., 8:364-371).

Other growth factors/hormones besides TGF and BMP can be used in the practice of the invention to influence new bone formation following fracture. For example, fibroblast growth factor injected into a rat fracture site (Jingushi *et al.*, 1990) at multiple high

doses (1.0 mg/50 ml) resulted in a significant increase in cartilage tissue in the fracture gap, while lower doses had no effect.

Calcium regulating hormones such as parathyroid hormone (PTH) also may be used in one aspect of the invention. PTH is an 84 amino acid calcium-regulated hormone whose principal function is to raise Ca^{+2} concentration in plasma and extracellular fluid. Studies with the native hormone and with synthetic peptides have demonstrated that the amino terminus of the molecule (amino acids 1-34) contains the structural requirements for biological activity (Tregear *et al.*, 1973; Hermann-Erlee *et al.*, 1976, Endocrine Research Communications, 3:21-35; Riond, 1993, Clin. Sci., 85:223-228).

Using a retroviral approach, a human full-length PTH gene construct has been introduced into cultured rat fibroblasts to create recombinant PTH-secreting cells. These cells then were transplanted into syngeneic rat recipients that were observed to develop hypercalcemia mediated by the increased serum concentrations of PTH (Wilson *et al.*, 1992). The object of these studies was to create an animal model of primary hyperparathyroidism.

PTH has a dual effect on new bone formation, a somewhat confusing aspect of hormone function despite intensive investigation. PTH has been shown to be a potent direct inhibitor of type I collagen production by osteoblasts (Kream *et al.*, 1993). Intact PTH also was shown to stimulate bone resorption in organ culture over 30 years ago, and the hormone is known to increase the number and activity of osteoclasts. It currently is accepted that osteoclast activation occurs via an osteoblast signaling mechanism.

On the other hand, osteosclerosis may occur in human patients with primary hyperparathyroidism (Seyle, 1932). It is well known that individuals with hyperparathyroidism do not inexorably lose bone mass, but eventually achieve a new bone remodeling steady state after an initial period of net bone loss. Chronic, low dose administration of the amino-terminal fragment of PTH (amino acids 1-34) also can induce new bone formation according to a time- and dose-dependent schedule (Seyle, 1932; Parsons & Reit, 1974).

Human PTH1-34 recently has been shown to: (a) stimulate DNA synthesis in chick osteoblasts and chondrocytes in culture (van der Plas, 1985; Schluter *et al.*, 1989; Somjen *et al.*, 1990); (b) increase bone cell number in vivo (Malluche *et al.*, 1986); (c) enhance the in vitro growth of chick embryonic cartilage and bone (Kawashima, 1980; Burch & Lebovitz, 1983; Lewinson & Silbermann, 1986; Endo *et al.*, 1980; Klein-Nulend *et*

al., 1990); (d) enhance surface bone formation (both cortical and trabecular bone) in normal and osteogenic animals and in humans with osteoporosis (Reeve *et al.*, 1976; Reeve *et al.*, 1980; Tam *et al.*, 1982; Hefti *et al.*, 1982; Podbesek *et al.*, 1983; Stevenson & Parsons, 1983; Slovik *et al.*, 1986; Gunness-Hey & Hock, 1984; Tada *et al.*, 1988; Spencer *et al.*, 1989; Hock & Fonseca, 1990; Liu & Kalu, 1990; Hock & Gera, 1992; Mitlak *et al.*, 1992; Ejersted *et al.*, 1993) and (e) delay and reverse the catabolic effects of estrogen deprivation on bone mass (Hock *et al.*, 1988; Hori *et al.*, 1988; Gunness-Hey & Hock, 1989) Liu *et al.*, 1991). Evidence of synergistic interactions between hPTH1-34 and other anabolic molecules has been presented, including insulin-like growth factor, BMP-2, growth hormone, vitamin D and TGF-β (Slovik *et al.*, 1986; Spencer *et al.*, 1989; Mitlak *et al.*, 1992; Canalis *et al.*, 1989; Linkhart & Mohan, 1989; Seitz *et al.*, 1992; Vukicevic *et al.*, 1989).

Anecdotal observation has shown that serum PTH levels may be elevated following bone fracture (Meller *et al.*, 1984; Johnston *et al.*, 1985; Compston *et al.*, 1989; Hardy *et al.*, 1993), but the significance of this observation is not understood. There are apparently no reports in the literature concerning attempts to localize either PTH or the PTH/PTHrP receptor in situ in human fracture sites or in experimental models. Furthermore, no attempt has been made to augment bone repair by the exogenous addition of PTH peptides. Although hPTH1-34 was known to function as an anabolic agent for bone, prior to the present invention, much remained to be learned about the role (if any) of PTH during bone regeneration and repair.

The present inventors surprisingly have found that direct gene transfer into areas where bone repair and/or regeneration is necessary can be achieved readily with the use of viral vectors containing a bone growth factor gene of interest under the control of a tissue specific promoter, such as the osteocalcin promoter. Currently, the preferred methods for achieving gene transfer generally involve using a fibrous collagen implant material soaked in a solution of DNA shortly before being placed in the site in which one desires to promote bone growth or using a preparation of plasmid DNA encapsulated in a synthetic matrix such as a block copolymer of PLGA. As the studies presented herein show, no implant material is necessary to facilitates the targeted uptake of exogenous viral constructs by cells which participate in bone regeneration/repair.

Several additional clinical applications of the present invention include, but are not limited to, the use of the subject vectors in spinal fusions, compression fractures and the stabilization of implant devices.

1. **Spinal Fusions**

The need for long term spinal stability is a frequent requirement in neurosurgical patients. In the cervical spine, anterior or posterior subluxation, especially at the C1-C2 level, is frequently encountered and often produces myelopathic symptoms, including a spastic gait and weakness. Progressive chronic instability may require surgical reduction with fusion, such as occiput to C2 or C3 fusion, posterior C1-C2 fusion or anterior cervical corpectomy with bone graft fusion. In the lumbar spine, degenerative changes including ligamentum hypertrophy, facet hypertrophy, disk bulging and subluxation can lead to lumbar stenosis causing neurogenic claudication. The symptomatology of lumbar stenosis includes weakness, numbness and pain, especially while walking and standing. It typically is relieved by a posterior lumbar decompression. The indications for spinal fusions after lumbar decompressive approaches currently are being studied, but probably include scoliosis, degenerative spondylolisthesis and excessive facet removal. The present inventors surprisingly have demonstrated the utility of bone growth factors to improve the rate and amount of bone deposition at spinal fusion sites, supporting the role of gene therapy to deliver bone growth factors to these regions to ensure long term spine stabilization.

2. **Compression Fractures**

Osteoporosis is characterized histologically by the parallel loss of bone mineral and bone matrix. Its cause may be physiologic, such as post-menopausal estrogen deficiency, or may be a primary age related decline in bone mass. The current treatment of osteoporosis is far from satisfactory. Although prophylaxis is the most effective form of management, there are no therapeutic options currently available to reverse the loss of bone mass in advanced osteoporosis. Diphosphonates, which are slowly hydrolyzable diphosphate analogs which inhibit osteoclastic bone resorption, and fluoride therapy, which stimulates bone formation and can substantially increase vertebral bone mass, are both being evaluated for clinical efficacy. Since bone growth factors, *i.e.* bone morphogenetic proteins, are characterized by their ability to induce endochondral bone formation de novo, they are

certainly of potential use in this typically progressive disease. This applies to neurosurgery through treatment of vertebral compression fractures commonly seen in these patients. Thus, the compositions and methods of the present invention would be ideally suited for the treatment of compression fractures that typically arise in a patient suffering from osteoporosis.

3. Bone Morphogenetic Protein in the Stabilization of Implant Devices.

Spinal instrumentation can provide immediate stabilization. One of the most crucial aspects in spinal instrumentation in order to ensure long-term efficacy is to establish a strong, rapid prosthesis/bone interface. Most prosthetic devices are designed with surface modifications such as grooves or holes to provide a region for bony ingrowth. This osseointegration of the implant provides for long-term stability. Bone growth factors certainly are ideal candidates to increase the amount of bone apposition and/or ingrowth to the implant, allowing for early and long term prosthesis stabilization. Cook *et al* demonstrated that BMPs can enhance osseointegration of porous metal implants and significantly can increase the amount of bone deposition at the implant-bone interface (Cook SD, *et al.*, 1996, Clinical Orthopedics and Related Research 324:29-38). In addition, these investigators demonstrated that BMP coated dental implants increase new bone formation adjacent to the implant surface. Certainly, the use of bone morphogenetic proteins delivered either locally or via gene therapy could increase the efficacy of these treatment modalities and decrease post-operative complication rates, such as implant extrusion, osteonecrosis, bone erosion and fractures.

Further studies are presented herein demonstrating that the transfer of an osteotropic gene results in cellular expression of a recombinant osteotropic molecule, which expression is directly associated with stimulation of new bone formation. Specifically, a gene transfer vector coding for BMP-2 will stimulate new bone formation.

VI. EXAMPLE 1: Construction of Recombinant Ad-OC-BMP-2 Vector

In order to target BMP gene expression to osteoblasts, an adenoviral vector containing the OC promoter to drive the BMP-2 transgene was constructed. Briefly, to make recombinant adenovirus with BMP-2 under control of the human OC promoter, the cytosine

deaminase gene in the El shuttle vector, pΔElspl-OC-CD (a gift from Dr. Kao), was replaced with the coding sequence of the human BMP-2. This was followed by recombination of sequences in the new shuttle vector with the master Ad5 plasmid, JM17, which contains the backbone of the circular adenovirus Ad5dl309 genome. In order to verify the adenovirus construct, an alkaline lysis viral DNA prep was made using an aliquot of the primary stock and PCR-amplified using AmpliTaq Gold (Perkin Elmer Corporation, Norwalk, CT) to verify that the adenovirus contained the BMP-2 sequence. Primers (Integrated DNA Technologies, Coralville, IA) designed to give a 529 bp product were:
forward: 5'-CCAGGTTGGTGAATCAGAATGC and
reverse: 5'-GAGATAGCACTGAGTTCTGTCG. PCR was carried out under the following conditions: 10 min. at 95°C followed by 30 cycles of: 30 sec. denaturing at 95°C, 1 min. annealing at 56°C and 2 min. extension at 72°C; the reaction was finished by a final extension step of 7 min. at 60°C. A reaction with no template was run as a negative control and reactions with pBMP2-125 and pΔE1sp1-OC-BMP2 were run as positive controls. Amplification products were visualized on a 2% agarose gel.

VII. EXAMPLE 2: *In Vivo* Bone Formation Using Ad-OC-BMP-2

A. Materials And Methods

1. **Animals:** A total of twelve rats are used in this study. Because host immune responses may limit transgene expression in adenoviral vectors, athymic nude rats (Harlan Sprague Dawley, Inc.) are utilized in this study (Festin *et al.*, 1978). These rats do not respond to T-cell dependent mitogens present when adenoviruses infect normal cells.

2. **Adenoviral constructs:** The recombinant Ad-OC-BMP-2 vector described in Example 1, above is used to monitor bone formation. Another recombinant adenovirus type 5 with the β-galactosidase (β-gal) gene under the control of the CMV promoter (Ad-CMV-β-gal), is used as a control. Both of these adenoviruses were made replication defective through complete deletion of the Ela and Elb and partial deletion of the E3 regions of the viral genome. The viruses were propagated on 293 cells and purified by two rounds of cesium chloride centrifugation. The purified virus was dialyzed in PBS. The

virus are stored until use at -80°C in PBS, 10% glycerol at a concentration of 5×10^8 particles/μl.

3. **Surgery**: The twelve athymic nude rats are sedated with chloral hydrate and the thighs prepped in a sterile fashion. Using a 19 gauge guide needle, the skin 1 cm above the knee joint is punctured and the needle advanced 1 cm proximally. Through this needle, a Hamilton microsyringe is inserted and 5 μl of viral solution (2.5×10^9 particles) are injected. The Ad-OC-BMP-2 virus is injected into the right thigh and the Ad-CMV-β-gal virus into the left thigh.

4. **Radiology**: The animals are sedated and CT scans through the thigh are performed at weeks 3 and 5 post-injection. Scanning is performed using a Picker PQ-2000 (version 4.2; Picker International, Cleveland, OH). Axial images with a 1 mm collimation and 1 mm table increment are obtained using the standard algorithm with 130 kV, 100mA, a 2 second scan time and a 40 mm image size. Three dimensional reconstruction is performed using a Voxel Q workstation (Picker International).

5. **Histology**: At day 3 post-injection, 4 of the animals are sacrificed and the thigh muscles are harvested, fixed with 0.05% glutaraldehyde solution and stained with Xgal reagent made with 35 mM $K_3Fe(CN)_6$, 35 mM $K_4Fe(CN)_6$ and 2mM $MgCl_2$ (Sigma, St. Louis, MO) with 5-bromo-4-chloro-3-indolyl-β-D-galactoside at a concentration of 1 mg/ml (Boehringer Mannheim, Germany). The specimens are then dehydrated through a series of graded ethanols, xylol and, finally, xylene. The thigh is then infiltrated and embedded in paraffin. Using a microtome, the tissue is sectioned into 1 μm slices and mounted on treated slides. After drying overnight, some of the slides are counterstained with hematoxylin and eosin after which all of the slides are cover slipped. At week 6, the remaining six animals are sacrificed. After anesthetizing each animal, transcardial perfusion with 100 ml of phosphate buffered saline, followed by 350 ml of a 4.0% paraformaldehyde and 0.5% glutaraldehyde fixative solution is performed. Both thighs are harvested for histologic examination as outlined above. The slides are stained with Alcian blue (pH=2.5), nuclear fast red and hematoxylin and eosin.

B. Results

At about 3 days post-injection, β-gal expression will be seen in both muscle cells and fibroblasts. At about 5 weeks post-injection, the animals are sacrificed and histological analysis of each thigh muscle is obtained. The CT scans will show intramuscular, ectopic calcification at an increased percentage of the Ad-OC-BMP-2 injection sites compared with that for the control Ad-CMV-β-gal injection sites. In fact, almost no radiographic changes will exist at the control injection sites.

Analysis of the density of the bone ossicle is performed by measuring the Hounsfield Units (HU) at about 3 and 5 weeks post-injection. An increase in the bone density will be seen at 5 weeks as compared to that at 3 weeks. In addition, CT scanning through the abdomen, with special attention to the liver, will show no evidence of systemic ectopic calcification. Histologic sections through the Ad-OC-BMP-2 injection site at about six weeks post-injection will reveal evidence of endochondral bone formation, including primitive mesenchymal cells, cartilage and bone. Further, bone induction within the thigh muscles likely will be limited to the injection site.

C. Discussion

This study will demonstrate, both radiographically and histologically, that bone formation can be induced *in vivo* by using direct injection of an adenoviral OC-BMP-2 gene construct. The newly formed bone is limited to the injection site, without evidence of local or systemic toxicity. The study supports the concept that BMP-2 gene therapy is a viable approach to stimulate or enhance endochondral bone formation and could have a wide variety of clinical and research applications.

There are numerous pathologic conditions in which enhanced bone formation could be of therapeutic value. In models of calvarial defects, researchers have demonstrated that closure is promoted through the placement of BMPs (Miki and Imai, 1996; Sweeney *et al.*, 1995; Takagi and Urist, 1982b). Several previous pre-clinical studies have demonstrated that BMPs can induce bone formation during spinal fusion procedures (Cook and Rueger, 1996; Helm *et al.*, 1997; Sandhu *et al.*, 1995; Schimandle *et al.*, 1996; Sheehan *et al.*, 1996). Sheehan *et al.* clearly demonstrated increased bone deposition, radiodensity and biomechanical strength at spinal fusion sites with the addition of rhBMP-2 to autologous bone grafts in a dog model. Substantial bony repair of femoral defects using the direct

application of BMPs also have been dramatically demonstrated in several species (Takagi and Urist, 1982a Yasko *et al.*, 1992). Through improving the osseointegration of prosthetic arthroplasty devices, BMPs have improved the stability and increased the longevity of artificial joints.

Several recent abstracts have demonstrated the use of Ad-BMP-2 in *ex vivo* models. These have involved *ex vivo* transfection of cells in tissue culture by Ad-BMP-2 and then placement of these transfected cells into rat thighs with subsequent bone formation. The present invention, however, is based upon the surprising discovery that direct injection of Ad-BMP-2 also results in endochondral bone formation.

The limitations of direct *in vivo* application of the rh BMPs include poor control of bone formation and the inability to deliver the protein systemically to treat diffuse bone diseases. The utilization of gene therapy techniques to express BMP-2 has several theoretical and practical advantages. Firstly, sustained BMP-2 gene expression at the treatment site would be possible using viral vectors such as Ad-OC-BMP-2. For example, since adenoviral vectors typically provide transgene expression for several weeks, improved bone formation may be possible compared to a single rh BMP treatment. In addition, a lentiviral delivery of the BMP-2 gene may produce stably transfected cells, which could be useful for the treatment of chronic, progressive osteopenic states, such as osteoporosis. Secondly, modifications of the viral construct, as taught in the present application, would make it possible to target BMP-2 gene expression to specific cells or tissues. These techniques could include genetically altering the viral coating to establish tissue specific transduction or utilizing tissue specific promoters, such as the OC promoter, to drive transgene expression. These approaches also make it possible to deliver the BMP-2 vector systemically, while maintaining tissue specific BMP-2 gene expression. Lastly, the utilization of inducible promoters, such as a tetracycline responsive promoter, may make it possible to up-regulate or down-regulate BMP-2 production *in vivo* after successful gene delivery. Gossen, M., *et al.*, 1992, Proc. Natl. Acad. Sci., 89:5547-5551; Miller M, *et al.*, 1997, Human Gene Ther., 8:803-815. Thus, regulating bone formation using gene therapy techniques will have significant clinical applications, especially in chronic bone diseases.

Since Ad-OC-BMP-2 induces bone formation only at the site of vector injection, without evidence of diffuse toxicity, the present technique has several clear clinical applications. The adenoviral vector could be applied to fracture sites to stimulate

osteoinduction, improving the rate and strength of bone healing. Addition of the Ad-OC-BMP-2 vector to autologous bone grafts during spine stabilization procedures may improve their clinical efficacy by improving osteointegration of the graft and also may decrease the amount of bone graft required. In addition, direct injection of the adenoviral construct alone adjacent to the spine may lead to long-term spine arthrodesis, eliminating the need for an open spine procedure. As mentioned previously, the addition of an inducible promoter to the construct could be used to control the rate and amount of bone deposition at sites of bone healing, which certainly would be of practical benefit to ensure adequate bone formation and to prevent excessive bony overgrowth.

Bone morphogen gene therapy also may be useful in the treatment of several genetic diseases which probably involve BMP overexpression, including autosomal dominant fibrodysplasia ossificans progressiva and myositis ossificans. Gannon *et al.* clearly demonstrated in an immunohistochemical study that BMP-2 and BMP-4 are present at increased levels in early fibromatous lesions in fibrodysplasia ossificans progressiva (Gannon *et al.*, 1997). In addition, Shafritz *et al.* demonstrated using Northern blot analysis and ribonuclease protection assays that BMP-4 mRNA levels are increased in lymphoblastoid cell lines from patients with this progressive disease (Shafritz *et al*, 1996). Thus, anti-sense gene therapy, for example, in these patients could be utilized to inactivate the over-expressed mRNA, leading to a successful treatment for this devastating condition.

VIII. EXAMPLE 3: Paraspinal Bone Formation Using Ad-OC-BMP-2

A. Materials And Methods

1. **Animals and Constructs:** The same animals and constructs described in Example 2, above are used for this example.

2. **Surgery:** The twelve athymic nude rats are sedated with a mixture of ketamine and xylazine, and the lumbosacral area is prepared in a sterile fashion. The rats, divided into three groups of four animals each, undergo paraspinal, percutaneous injection at the lumbosacral junction with 7.5 µl of virus. The three groups are as follows: 1) bilateral injection of Ad-OC-BMP-2, 2) bilateral injection of Ad-CMV-β-gal, and 3) injection of Ad-

OC-BMP-2 on the right and injection of Ad-CMV-β-gal on the left. To place the virus in the proper location, a 19 gauge guide needle is inserted in the junction of the spinous process and lamina on each side. Through this needle, a Hamilton microsyringe is placed and 7.5 μl of virus is injected. This procedure is repeated on the other side. Separate guide needles and syringes are used for the two different viruses.

3. **Radiology**: The animals are sedated and CT scans of the lumbosacral junction are obtained at weeks 3, 5, 8 and 12 post-injection. Scanning is performed using a Picker PQ-2000 (version 4.2; Picker International, Cleveland, OH). Axial images with a 1 mm collimation and 1 mm table increment are obtained using the standard algorithm with 130 kV, 100mA, a 2 second scan time and a 40 mm image size. Three dimensional reconstruction is performed using a Voxel Q workstation (Picker International).

4. **Histology**: At 12 weeks post-injection, the rats are killed and undergo transcardial perfusion with 100 ml PBS followed by 350 mls 0.05% glutaraldehyde solution. The lumbosacral spines are removed and decalcified with decalcifying solution composed of 10% HCl and 0.1% ethylenediaminetetraacetic acid. The specimens are then dehydrated through a series of graded ethanols, xylol and, finally, xylene after which the spines are infiltrated and embedded in paraffin. Using a microtome, the tissue is sectioned into 10 μm slices and mounted on treated slides. After drying overnight, some of the slides are stained with Alcian blue (pH=2.5), nuclear fast red and hematoxylin and eosin, after which, cover slips are placed over all slides.

B. Results

At about 3, 5, 8 and 12 weeks post-injection, the CT scans will show intramuscular, ectopic calcification at an increased percentage of the Ad-OC-BMP-2 injection sites compared with that for the control Ad-CMV-β-gal injection sites. In fact, almost no radiographic changes will exist at the control injection sites.

Analysis of the density of the bone ossicle is performed by measuring the Hounsfield Units (HU). An increase in the bone density will be seen over time. In addition, the spinal canal will show no evidence of ectopic calcification within and no growth of bone from outside in.

Histological examination of sections of the Ad-OC-BMP-2 injection site 12 weeks post-injection will show extensive endochondral bone formation within the paraspinal musculature. There will be large areas of mature bone that contain developed vascular channels as well as areas of cartilage. The new bone will be in solid continuity with the adjacent laminae, the inferior facet and spinal process. Further, the focal bone deposition will have sharp borders, but will show no evidence of extensive diffusion into the surrounding muscle or spinal cord.

C. Discussion

This study will demonstrate, both radiographically and histologically, that gene therapy techniques can be used to cause expression of BMP-2 in the paraspinal region and lead to endochondral bone formation. Further, there is no evidence of bone formation distant from the injection site and no evidence of neural compromise, suggesting that this approach will be safe to use in a clinical setting. Thus, this example again shows that bone formation can be induced *in vivo* by using direct injection of an adenoviral OC-BMP-2 gene construct.

This novel gene therapy technique may have numerous applications in spine stabilization procedures. The Ad-OC-BMP-2 vector could be added to autologous bone grafts to enhance bone deposition at the fusion site. Therefore, BMP-gene therapy could have the potential to improve long-term stability significantly after spine instrumentation and limit the need for extensive harvesting of autograft from the iliac crest or fibula. Alternatively, percutaneous injection of the vector into the paraspinal musculature, facet joints, or the annulus fibrosis could fuse the spine in a minimally invasive fashion. In addition, percutaneous injection of the vector into spinal fractures could increase the rate of bone repair and decrease the risk of nonunion and spinal instability. In summary, this example demonstrates the feasibility of utilizing direct, percutaneous injections of adenoviral OC-BMP-2 to induce spinal arthrodesis.

Although the invention is described in detail with reference to specific embodiments thereof, it will be understood that variations which are functionally equivalent are within the scope of this invention. Indeed, various modifications of the invention in addition to those shown and described herein will become apparent to those skilled in the art

from the foregoing description and accompanying drawings. Such modifications are intended to fall within the scope of the appended claims.

Various publications are cited herein, each of the disclosures of which is incorporated by reference in its entirety.

WHAT IS CLAIMED IS:

1. A method for promoting bone repair comprising administering a polynucleotide to an area where bone repair is necessary, wherein the polynucleotide encodes an osteotropic factor operatively linked to a bone specific promoter.

2. The method of claim 1, wherein the osteotropic factor is a bone morphogenic protein, a transforming growth factor, a fibroblast growth factor or an endothelial growth factor.

3. The method of claim 2, wherein said osteotropic factor is bone morphogenic factor.

4. The method of claim 1, wherein the bone specific promoter is the osteocalcin promoter.

5. The method of claim 1, wherein the bone specific promoter is an inducible promoter.

6. The method of claim 1, wherein the polynucleotide comprises more than one osteotropic factor.

7. The method of claim 1 wherein the administering is by an intravenous route or a direct injection of the polynucleotide..

8. A method for promoting bone repair comprising administering a polynucleotide to an area where bone repair is necessary wherein the polynucleotide encodes a ribozyme or an antisense RNA.

9. A polynucleotide encoding an osteotropic factor operatively linked to a bone specific promoter.

10. The polynucleotide of claim 9, wherein the osteotropic factor is a bone morphogenic protein, a transforming growth factor, a fibroblast growth factor or an endothelial growth factor.

11. The polynucleotide of claim 10, wherein said osteotropic factor is bone morphogenic factor.

12. The polynucleotide of claim 9, wherein the bone specific promoter is the osteocalcin promoter.

13. The polynucleotide of claim 9, wherein the bone specific promoter is an inducible promoter.

14. The polynucleotide of claim 9, wherein the polynucleotide comprises more than one osteotropic factor.

15. A polynucleotide encoding a ribozyme or an antisense RNA, wherein the polynucleotide promotes bone repair.

16. A therapeutic agent comprising a recombinant viral vector containing a polynucleotide encoding an osteotropic factor operatively linked to a bone specific promoter.

17. The therapeutic agent of claim 16, wherein the viral vector is a retroviral vector, an adeno-associated viral vector or an adenoviral vector.

18. The therapeutic agent of claim 16, wherein the viral vector is the adenoviral vector.

19. The therapeutic agent of claim 16, wherein the osteotropic factor is a bone morphogenic protein, a transforming growth factor, a fibroblast growth factor or an endothelial growth factor.

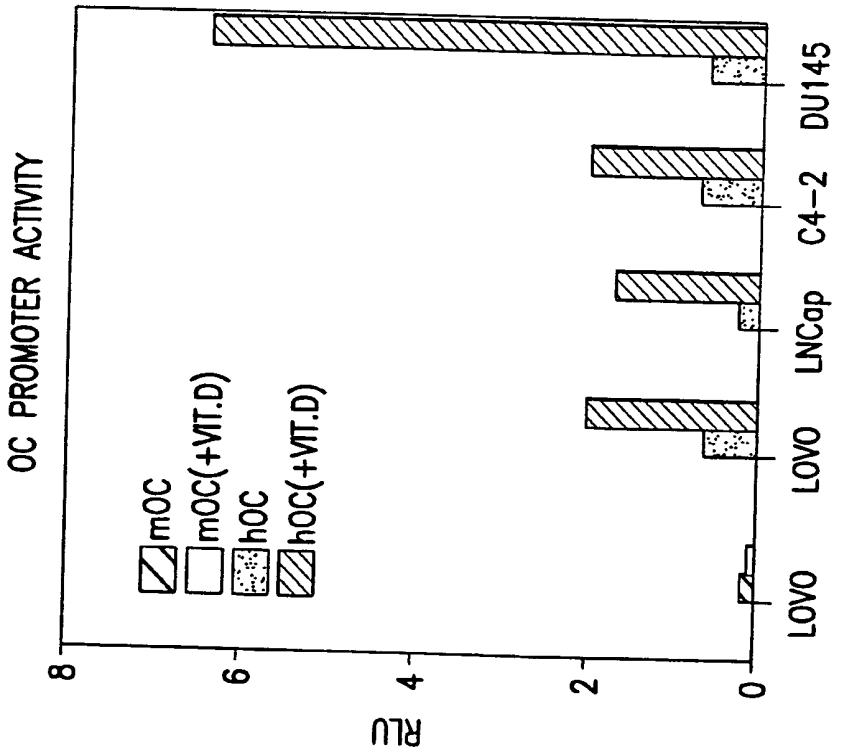
20. The therapeutic agent of claim 16, wherein said osteotropic factor is bone morphogenic factor.

21. The therapeutic agent of claim 16, wherein the bone specific promoter is the osteocalcin promoter.

22. The therapeutic agent of claim 16, wherein the bone specific promoter is an inducible promoter.

23. The therapeutic agent of claim 16, wherein the polynucleotide comprises more than one osteotropic factor.

24. A therapeutic agent encoding a ribozyme or an antisense RNA, wherein the therapeutic agent promotes bone repair.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mOC(+VIT.D)
hOC
hOC(+VIT.D)
RLU
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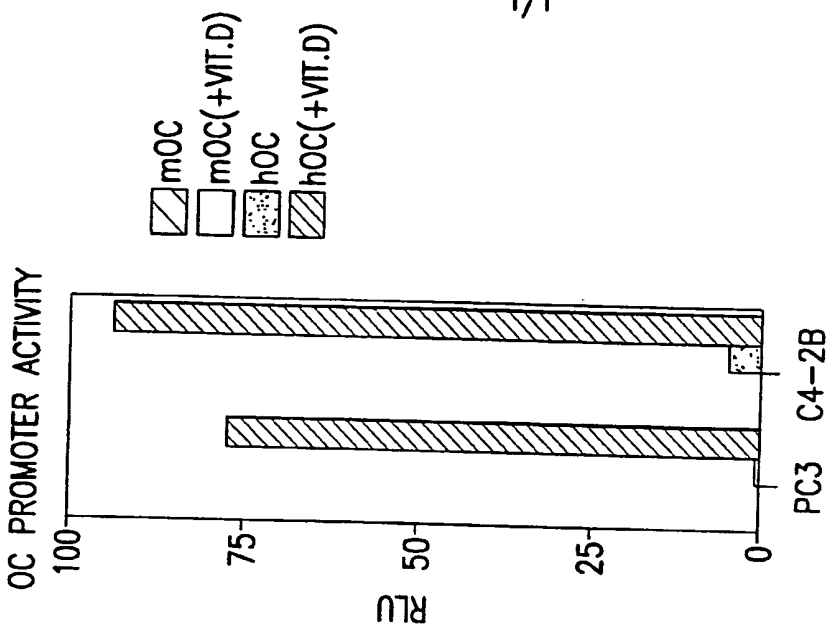
2
4
6
8
LOVO
LOVO
LNCap
C4-2
DU145
CELL TYPE

FIG.1A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hOC(+VIT.D)
RLU
0
25
50
75
100
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CELL TYPE

FIG.1B